UTILITY OF CLAIMS 1-10 and 18-37 UNDER 35 U.S.C. §101 AND §112, 1st ¶

Claims 1-10 and 18-37 stand rejected as lacking a well established utility under §§ 101 and 112, first paragraph. This rejection is respectfully traversed.

Applicants set forth in the specification many utilities for the claimed antibodies, as well as teachings sufficient to enable one of skill in the art to make and use the antibodies. Nonetheless, the Patent and Trademark Office rejects the claims because:

- "the specification offers little information about the actual existence of the TEM 17 protein [to which the claimed antibodies bind] or its functional role" (Paper No. 013122004 at page 3, lines 17-18);
- "no data with regard to its [TEM 17's] actual existence as a protein has been offered" (Paper No. 03122004 at page 4, lines 4-5);
- "there is no proof that the protein has actually been translated" (Paper No. 03122004 at page 4, lines 21-22);
- "differential expression of the protein would be required for use in either application[s]" (Paper No. 03122004 at page 5, lines 1-2).

"Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility." *In re Brana,* 51 F.3d 1560, 34 U.S..P.Q.2d 1436 (Fed. Cir. 1995) (citing In re Bundy, 642 F.2d 430, 433, 209 U.S.P.Q. 8, 51 (C.C.P.A. 1981)).

Although applicants do not concede that the Patent and Trademark Office has shifted the burden of proof of utility to applicants, to expedite prosecution applicants present evidence in the enclosed Declaration of Kenneth W. Kinzler which demonstrates that:

- TEM 17 protein is produced in human tumor tissue (paragraph 5);
- TEM 17 protein is differentially expressed between tumor tissue and normal tissue (paragraphs 5 and 6);
- Anti-TEM 17 antibodies specifically label tumor vessels *in vivo*; and
- TEM 17 protein specifically binds to cortactin, a protein which is involved in cell migration and the cytoskeleton (paragraph 7).

These data demonstrate that TEM 17 is expressed in human tissue, that TEM 17 is differentially expressed in tumor versus normal tissue, and that TEM 17 binds to a protein known in the art to be involved in cell migration and the cytoskeleton. These data support the real world utilities taught in the specification for anti-TEM 17 antibodies, namely:

- to inhibit neoangiogenesis;
- to inhibit tumor growth;
- to isolate endothelial cells; and
- to identify tumor endothelium for diagnosis.

"After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of evidence with due consideration to persuasiveness of argument." *In re Oetiker,* 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). An applicant can do this using any combination of the following: amendments to the claims, arguments or reasoning, new evidence submitted in an affidavit or declaration under 37 CFR 1.132, or in a printed publication. New evidence provided by an applicant must be relevant to the issues raised in the rejection. M.P.E.P. § 2107.02, VI.

Only where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained. If the record as a whole would make it more likely than not that the asserted utility for the claimed invention would be considered credible by a person of ordinary skill in the art, the Office cannot maintain the rejection. *In re Rinehart,* 531 F.2d 1048, 1052, 189 U.S.P.Q. 143, 147 (C.C.P.A. 1976).

In view of the data provided in Dr. Kinzler's declaration, one of ordinary skill in the art would find the asserted utilities real, substantial, and credible.

Withdrawal of these dual rejections under § 112[1] and § 101 is respectfully requested.

REJECTION OF CLAIMS 1-2 AND 32-33 UNDER §102(b)

Claims 1-2 and 32-33 are rejected as anticipated by Jacobs WO 98/14576. Claims 1 and 2 are directed to intact antibodies or other molecules comprising an antibody variable region which specifically binds to an extracellular domain of TEM 17 as shown in SEQ ID NO:230. Claims 32 and 33 specify that the extracellular domain is amino acid residues 18-427 of TEM 17.

"To anticipate, every element and limitation of the claimed invention must be found in a single prior art reference, arranged as in the claim." *Brown v. 3M,* 265 F.3d 1349, 1351, 60 U.S.P.Q.2d 1375, 1376 (Fed. Cir. 2001).

> [R]ejections under 35 U.S.C. 102 are proper only when the claimed subject matter *is* identically disclosed or described in the 'prior art.' Thus for the instant rejection under 35 U.S.C. 102 . . . to have been proper, the . . . reference must clearly and unequivocally disclose the claimed compound or direct those skilled in the art to the compound without *any*

1 Applicants assume that the separate "written description" rejection has been withdrawn, as it has not been repeated in Paper No. 03122004.

> need for picking, choosing, and combining various disclosures not directly related to each other by the teachings of the cited references. Such picking and choosing . . . has no place in the making of a 102 anticipation rejection.

In re Arkley, 455 F.2d 586, 587, 172 U.S.P.Q. 524, 526 (C.C.P.A. 1972) (emphasis in original).

Jacobs is cited as anticipating the antibodies recited in claims 1, 2, and 32-33, *i.e.*, antibodies that specifically bind to residues 18-427 or the extracellular domain of TEM 17 (SEQ ID NO:230). However, Jacobs taken as a whole does not teach such antibodies. Jacobs does not teach each and every limitation of the claim, arranged as in the claim. Only by picking and choosing among portions of Jacobs' disclosure and combining them in a way that Jacobs does not could one find and arrange the elements of the claimed antibodies as recited in claims 1, 2, 32, and 33.

Jacobs generically teaches using immunization techniques to raise antibodies (page 36, lines 22-24) to recombinant proteins expressed by the disclosed polynucleotides (page 36, lines 11-12).

What recombinant protein relevant to the claimed subject matter does Jacobs teach? Jacobs teaches a predicted amino acid sequence from clone CC194-4 that has amino acids 1 to 100 as its leader/signal sequence and that has a mature amino acid sequence beginning at amino acid 101. "Amino acids 1 to 100 are the predicted leader/signal sequence with the predicted mature amino acid sequence beginning at amino acid 101." Page 26, lines 1-4. A signal sequence is cleaved during synthesis of a protein and does not form part of a mature protein. See Alberts et al., *The Molecular Biology of the Cell,* pp. 343-344, Garland Publishing, Inc., New York, 1983 (appendix 1), http://www.cytochemistry.net/cell-biology/rer2.htm (appendix 2), and Walter and Blobel, *Proc. Natl. Acad. Sci. USA,* vol. 77, pp. 7112-7116, 1980 (appendix 3).

Jacobs did not actually express a protein from clone CC194_4; rather, he predicted a protein and predicted its attributes. Based on the teachings of Jacobs, if one of skill in the art were to raise antibodies to the predicted protein of CC194_4, he would make an expression construct encoding the mature protein, *i.e.*, amino acids residues 101 and following. He would not make an expression construct to express the portion of the sequence which Jacobs explicitly teaches is *removed* from the protein. Thus, one of skill in the art would synthesize or express amino acid residues 101-108 and raise antibodies to that. Amino acid residues 101-108 of Jacobs correspond to amino acid residues 443-450 of TEM 17 or SEQ ID NO: 230. These amino acid residues are not, however, in the extracellular domain of TEM 17 as required by claims 1 and 2. These amino acid residues are not within residues 18-427 of TEM 17 as required by claims 32 and 33. See alignment submitted with applicants' prior amendment.

Thus, even if, *arguendo*, one of skill in the art were to follow assiduously the teachings of Jacobs to make the protein and antibodies which Jacobs himself did not make, he would not arrive at the claimed invention. Rather, he would arrive at an antibody to a polypeptide which is not part of TEM 17's extracellular domain.

One must consider a reference as a whole for all that it teaches. One cannot pick and choose among the teachings of a reference and ignore those which are inconvenient. See *In re Arkley, supra.* The Patent and Trademark Office, however, has ignored Jacobs' teaching regarding the identity of the mature protein. One of skill in the art would follow all of Jacobs' relevant teachings and not ignore certain portions. When making an antibody, one of skill in the art would not utilize a signal sequence that does not form part of the mature protein. Following all of Jacobs' teachings would not yield the present invention. Thus Jacobs does not anticipate

the present invention because Jacobs does not teach antibodies as recited in claims 1, 2, 32, and 33.

Withdrawal is respectfully requested.

Respectfully submitted,

Dated: August 18, 2004

By: Sarah A. Kagan

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Membrane-bound Ribosomes Are Derived from Free Ribosomes That Are Directed to the ER Membrane by Special Signal Sequences[19]

Although the membrane-bound ribosomes of the rough ER synthesize a selected class of proteins destined for vectorial discharge, there is no evidence that the ribosomes themselves differ from those free in the cytoplasm. In the current view, membrane-bound ribosomes are simply ribosomes that are specifically directed to the ER membrane because of the polypeptide chain they happen to be translating.

An important insight into how ribosomes are directed to the ER came from detailed studies of antibody (immunoglobulin) synthesis. The light and heavy chains of immunoglobulin constitute the principal secretory products of the plasma cells of the immune system (Chapter 17). When mRNA encoding the light chain of an immunoglobulin is translated *in vitro* by free ribosomes, the light chain is synthesized in the form of a precursor containing an extra 20 amino acid residues not found in the secreted product. The extra "leader" peptide is located at the extreme amino terminus of the precursor immunoglobulin. But chains synthesized *in vitro* by ribosomes attached to rough microsomes lack this extra amino-terminal peptide. These discoveries provided evidence for an earlier proposal that the *leader sequence* peptide acts as a "signal" to direct the ribosome to the rough regions of the ER membrane (Figure 7–28). Some receptor that recognizes proteins containing this *signal peptide* must therefore be present exclusively in the rough regions of the ER membrane.

This viewpoint has been greatly fortified by the subsequent discovery that the major secretory proteins of the pancreas, when synthesized *in vitro* on free ribosomes, are translated as precursor polypeptides ("presecretory" proteins) containing a similar amino-terminal leader sequence of 16 residues not found *in vivo*. Since then, precursors to dozens of secretory and integral membrane proteins in procaryotes and eucaryotes have been described. In all of these cases, the amino-terminal **signal sequence** contains large numbers of hydrophobic amino acid residues, even though the actual sequences

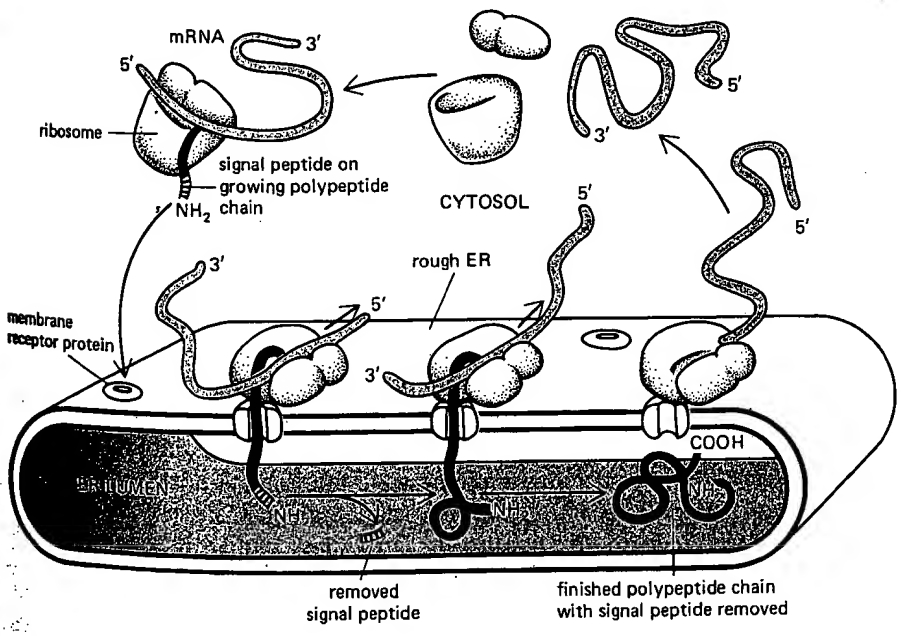

Figure 7–28 A schematic view of the signal peptide hypothesis. For simplicity, only a single ribosome, rather than a polyribosome, is shown. The indicated binding of a cytosolic ribosome to the rough ER is now thought to require two separate interactions: an affinity of the ribosome itself for special membrane proteins in the ER (presumably the ribophorins, see p. 341) and the interaction of an amino-terminal signal peptide on the growing polypeptide chain with a membrane receptor protein. However, once protein transfer across the membrane begins, the growing polypeptide chain itself helps to anchor the ribosome, replacing the second interaction.

are quite variable. It has now been shown that these signal sequences are, in fact, removed on the luminal side of the rough ER, even before the synthesis of the polypeptide is completed, by a specific protease found only in rough microsomes. This type of amino-terminal sequence that is cleaved off after protein synthesis has never been found on a protein that remains in the cytosol.

Ribosomes in the cytosol that begin synthesizing a protein destined for the ER must be brought to the rough ER to begin the vectorial discharge process. But how are these ribosomes specifically recognized by the ER membrane? Recent evidence has shown that a multisubunit protein present in the cytosol binds to these ribosomes shortly after they have synthesized the appropriate signal peptide. This protein, called *signal recognition protein,* halts further protein synthesis by the ribosome until it has become linked to the rough ER membrane. Presumably, this protein recognizes both the amino terminus of the newly synthesized protein and some receptor on the rough ER membrane, thereby binding the ribosome to the ER. Protein synthesis by the ribosome then resumes, as vectorial discharge begins. While the nature of the receptor in the ER membrane is unknown, the ribophorins are thought to be involved in stabilizing the ribosome-ER complex.

There Is Genetic Evidence for the Signal Hypothesis[20]

Genetic analysis of procaryotes has been used in two different ways to test the idea that leader sequences act as the proposed signals for the vectorial discharge of proteins. (1) Many mutants of an *E. coli* membrane protein have been isolated in which the protein is retained in the cytoplasm instead of being inserted in the membrane. Most of these mutations have been found to alter the extreme amino-terminal leader sequence region of the protein. (2) In specially designed strains of *E. coli,* the DNA segments coding for polypeptide chains of integral membrane proteins and of cytosolic proteins have been fused to create new hybrid proteins. When a substantial length of the amino-terminal portion of a membrane protein of *E. coli* is fused with most of the carboxyl-terminal portion of a cytosolic protein, the hybrid protein is found in the membrane. Thus, only the amino-terminal portion seems to be required for passage into a membrane.

Experiments in *E. coli* using hybrid proteins have demonstrated another important fact about signal sequences: although these sequences are required for directing the growing polypeptide to the membrane, they are not always sufficient in themselves. A hybrid protein containing only a very short length of the amino terminus of a membrane protein—including all of its leader sequence—was not inserted into the membrane. Thus, the leader sequence may normally interact with neighboring regions of the polypeptide chain to create a unique three-dimensional arrangement of amino acids, and it is this arrangement—rather than the leader sequence itself—that is required for specific membrane insertion.

Some Proteins Cross Membranes by a Posttranslational Import Mechanism Rather Than by Vectorial Discharge[21]

Some proteins can cross certain membranes long after their synthesis on ribosomes. For example, most of the proteins inside mitochondria and chloroplasts are coded for by nuclear genes (Chapter 9). These proteins are synthesized and released in the cytosol and then are pumped across the appropriate organelle membrane in an energy-requiring process (**posttranslational import**). Interestingly, most of these imported proteins also contain amino-terminal leader sequences that are removed following transport across the mitochondrial or chloroplast membranes. These leader sequences have been

MOLECULAR BIOLOGY OF
THE CELL

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Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

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"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson
The Cell in Development and Heredity
3rd edition, 1925, Macmillan, Inc.

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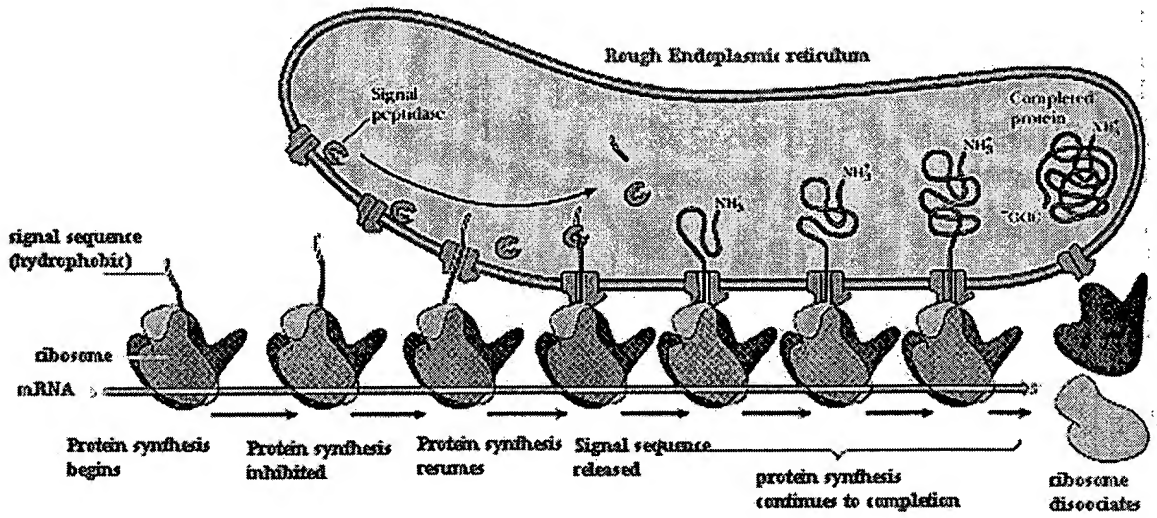
How do proteins translocate into the lumen of the rough endoplasmic reticulum?



What is different about the protein that is destined for the rough endoplasmic reticulum?

[Note: This section describes work that led to a Nobel Prize in Medicine and Physiology to Dr. Gunter Blobel. For more information about Dr. Blobel's work and the pioneering discoveries, click: http://www.nobel.se/medicine/laureates/1999/]

The major difference is the fact that it has a hydrophobic signal sequence. This simplified cartoon shows that this is the first part of the protein produced. After the signal sequence is completed, protein synthesis is further inhibited. This is to allow the interaction of the signal sequence with a complex on the rough endoplasmic reticulum. In the above cartoon, note that the signal peptide is allowed to enter and essentially guide the protein into the lumen of the rough endoplasmic reticulum. Once the signal sequence is detected, protein synthesis resumes and the rest of the protein is inserted in the lumen. Note that a signal peptidase near the inner surface of the membrane works to cleave the signal sequence from the growing peptide.

[***The text reading for this discussion is Alberts et al, Molecular Biology of the Cell, third edition, Garland Publishing, 1994, pp 577-588 (Chapter 12) and pp 599-616.*** *All of the figures in these web pages are linked to a page listing the citation from which the figure was taken. Click on the figure to learn the citation. If there is no link, the figure came from our own collection* <img src="translation4.jpg" alt="translation4.jpg (54729 bytes)" align="left" hspace="10" vs

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Biochemistry

Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum

(*in vitro* protein synthesis/salt extract of microsomal membranes/hydrophobic chromatography/reconstituted translocation activity/*N*-ethylmaleimide labeling)

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Communicated by Fritz Lipmann, August 27, 1980

ABSTRACT **The capacity of microsomal membranes to translocate nascent presecretory proteins across their lipid bilayer can be largely abolished by extracting them with high ionic strength buffers. It can be reconstituted by adding the salt extract back to the depleted membranes [Warren, G. & Dobberstein, B. (1978) *Nature (London)* 273, 569–571]. Utilizing hydrophobic chromatography, we purified to homogeneity a protein component of the salt extract that reconstitutes the translocation activity of the extracted membranes. This component behaves as a homogeneous species upon gel filtration, ion-exchange chromatography, adsorption chromatography, and sucrose-gradient centrifugation. When examined by polyacrylamide gel electrophoresis in $NaDodSO_4$, six polypeptides with apparent M_r of 72,000, 68,000, 54,000, 19,000, 14,000, and 9000 are observed in about equal and constant stoichiometry, suggesting that they are subunits of a complex. The sedimentation coefficient of 11S is in good agreement with the sum of the M_r of the subunits. The M_r 68,000 and 9000 subunits label intensely with *N*-[^{3}H]ethylmaleimide. Thus, the reported sulfhydryl group requirement of the translocation activity in the unfractionated extract [Jackson, R. C., Walter, P. & Blobel, G. (1980) *Nature (London)*, 286, 174–176] may be localized to either or both the M_r 68,000 and 9000 subunits of the purified complex.**

The synthesis of secretory and several membrane proteins so far investigated is thought to be initiated on free ribosomes (1, 2). When the nascent polypeptide chain emerges from the ribosome, a specific section of it, the signal sequence, was proposed to interact with receptors in the endoplasmic reticulum (ER), triggering the formation of a ribosome–membrane junction and initiating the translocation process of the nascent chain into the cisternae of the ER (1, 2). In most cases the signal sequence is located at the NH_2-terminal portion of the nascent chain (2) and is cleaved by signal peptidase (3, 4), presumably on the luminal side of the ER (5, 6), concomitantly with elongation and translocation of the protein.

Little is known about the recognition and transport machinery that is involved in the translocation process. The translocation activity of the microsomal vesicles has been assayed directly, after extraction and reconstitution experiments. One approach used salt extraction (7); the other one used limited tryptic dissection (6) of the microsomes. In both cases, a water soluble fraction and a translocation-inactive membrane fraction were obtained that recombined to reconstitute the translocation activity. Both water soluble fractions have been shown to be functionally equivalent (8) (the trypsin-derived fraction reconstitutes salt-depleted membranes and vice versa) and both contain a sulfhydryl group, which is required for their activity (8).

We describe here the purification to homogeneity of the active component of the salt extract that reconfers translocation activity to the salt-extracted microsomal membrane vesicles.

METHODS

All preparative procedures were carried out at 0–4°C. Optical absorbance determinations were done in 1% $NaDodSO_4$. All glassware was siliconized. The 4 M KOAc stock solution was adjusted to pH 7.5.

Preparation of Microsomal Membranes. Rough microsomal membranes were prepared from freshly excised dog pancreas (9) as described (10), with the following modifications: all buffers contained 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. The rough microsomes were collected by centrifugation for 2.5 hr at 140,000 × g_{avg}. The resulting pellet of rough microsomes (RM) was resuspended by manual homogenization in a Dounce homogenizer (A-pestle) in buffer A (250 mM sucrose/50 mM triethanolamine·HOAc, pH 7.5 [$(EtOH)_3N$·HOAc]/1.0 mM dithiothreitol) to a concentration of 50 A_{280} units/ml.

Adsorbed ribosomes and proteins were removed by passing the membranes through a Sepharose C1-2B column (11) in a low-salt buffer [50 mM $(EtOH)_3N$·HOAc/0.5 mM $Mg(OAc)_2$/1 mM dithiothreitol]. RM (20 ml) were loaded on a 200-ml column (upward flow, 15 ml/hr). The turbid fractions were pooled, and the membranes were collected by centrifugation (15 min at 50,000 × g_{avg}). The resulting washed RM were resuspended in 20 ml of buffer A.

Extraction of Washed Microsomal Membranes. Ten milliliters of an ice-cold salt solution (1.5 M KOAc/15 mM $Mg(OAc)_2$ was slowly added to 20 ml of washed RM. The mixture was incubated for 15 min on ice. The membranes were sedimented for 1 hr at 120,000 × g_{avg} through a cushion of 0.5 M sucrose in buffer B [50 mM $(EtOH)_3N$·HOAc/500 mM KOAc/5 mM $Mg(OAc)_2$/1 mM dithiothreitol] and the resulting pellet of salt-extracted microsomes was resuspended in 20 ml of buffer B containing 250 mM sucrose. The supernatant fraction, not including the cushion, was recentrifuged (3.5 hr at 200,000 × g_{avg}) to deplete it of ribosomes (postribosomal salt extract).

Fractionation of the Salt Extract by Hydrophobic Chromatography. A 2-ml column of ω-aminopentyl-agarose (12) (5.7 μmol of 1,5-diaminopentane coupled per ml) was prewashed with 10 ml of 2 M KOAc and then equilibrated with 20 ml of buffer B. The postribosomal salt extract fraction (24 ml) was passed over the column (6 ml/hr). The column was then washed with 10 ml of buffer B and eluted with buffer C [50 mM $(EtOH)_3N$·HOAc/1 M KOAc/10 mM $Mg(OAc)_2$/1 mM dithiothreitol/0.05% Nikkol detergent]. As soon as buffer C ap-

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Abbreviations: ER, endoplasmic reticulum; RM, rough microsomes; $(EtOH)_3N$, triethanolamine; eq, equivalent; PRL, prolactin; prePRL, preprolactin.

peared in the eluent (detected by an abrupt change in drop size due to the presence of detergent), a 2-ml fraction was collected.

Cell-Free Protein Synthesis. Bovine pituitary RNA (10, 13) (0.2 A_{260} units per 25 μl of translation mix) was translated in a staphylococcal nuclease-treated wheat germ system (14, 15) (6 μl of wheat germ S23 per 25 μl of translation mix). The ions that were added with the various membrane and salt extract fractions were taken into account and compensated for to yield a final ion concentration of 150 mM KOAc and 2 mM $Mg(OAc)_2$. Whenever detergent-containing fractions were assayed, the membranes were added last after all of the other components were mixed.

Polyacrylamide gel electrophoresis in $NaDodSO_4$ of the different fractions and translation products was performed on Cl_3CCOOH-precipitated, dithiothreitol-reduced, and iodoacetamide-alkylated samples as described (1). Polyacrylamide gradient (10–15%) slab gels were employed throughout.

Definitions. Percentage processing is defined as cpm in prolactin (PRL)/[cpm in PRL + cpm in preprolactin (prePRL)]. It is used as a measure of the translocation activity of membranes added in subsaturating amounts (6).

One equivalent (eq) is the amount of a fraction (supernatant or membrane) that is derived from 1 μl of RM suspension at a concentration of 50 A_{280} units/ml. One eq is roughly derived from 1 mg of tissue (wet weight). One unit of translocation activity (U) (*i*) for a membrane fraction is the amount of membranes that gives the same amount of processing (i.e., translocation) as 1 eq of RM (which under the conditions of all of our assays yields 30% processing), and (*ii*) for a supernatant fraction is the amount of supernatant that has to be added back to 1 eq of the salt-extracted (i.e., inactive by itself) RM to restore activity to that of 1 eq of RM.

Materials. [^{35}S]Methionine (1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), [^{3}H]OAc^- (140 mCi/mmol), and *N*-[^{3}H]ethylmaleimide (525 mCi/mmol) were from New England Nuclear. Nikkol (octaethyleneglycol mono-*n*-dodecyl ether) was from Nikko Chemicals, Tokyo, Japan. ω-Aminoalkyl-agarose was from Sigma.

RESULTS

The translocation activity of microsomal membranes can be readily assayed in cell-free protein-synthesizing systems (6). If one translates bovine pituitary RNA in the absence of membranes, a larger precursor, prePRL, is synthesized (13). If one carries out the translation in the presence of microsomal membranes, the newly made secretory protein is translocated into the lumen of the vesicles, as judged by its resistance to externally added proteases. Coupled to the translocation process is the proteolytic processing by signal peptidase, presumably on the luminal side of the vesicles (6). Because this processing step is very efficient relative to translocation (no unprocessed prePRL is found inside the vesicles), the ratio of PRL to prePRL is a convenient measure of the translocation activity of the membranes (Fig. 1*A*).

Here we use the wheat germ cell-free translation system.

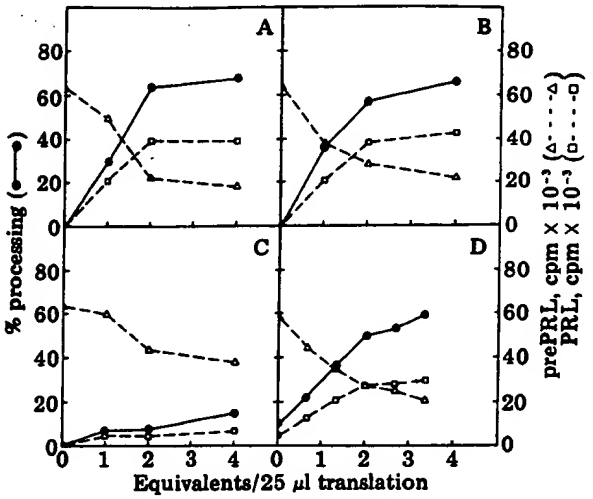

FIG. 1. Titration of membranes and crude salt extract in the translocation assay. Bovine pituitary RNA was translated in a 25-μl wheat germ system with increasing amounts of RM, washed RM, or salt extract added. The translation products were separated by polyacrylamide gel electrophoresis in $NaDodSO_4$. Bands corresponding to prePRL and PRL were located by autoradiography and sliced from the dried gel, and the radioactivity was determined (6). From the cpm values (△, prePRL; □, PRL) percentage processing was computed (●). (*A*) RM. (*B*) Washed RM. (*C*) Salt-extracted RM. (*D*) Increasing amounts of salt extract assayed in the presence of 1 eq of salt-extracted RM. All translations contained a final concentration of 0.002% Nikkol.

Under our conditions, mammalian polysomes still attached to the RM preparation do not read out in the plant translation system and, therefore, no "stripping" or nuclease treatment of the microsomes to deplete them of their endogenous mRNA activity is necessary.

As a first step toward purification prior to salt extraction, we freed our starting preparation of RM of adsorbed proteins and ribosomes by passing the membrane suspension over a Sepharose Cl-2B column in a low-salt buffer (11). Although this wash (Table 1) removed 40% of the A_{280} units (which otherwise would likely be coextracted by the high-salt buffer), it did not reduce the activity of the membrane fraction (Fig. 1*A* vs. 1*B*). Subsequent extraction by high-salt buffer depleted the membranes of most of their translocation activity (Fig. 1*C*). Recombining the salt extract with the depleted membranes restored their activity (Fig. 1*D*), as has been described (7, 8).

The activity of the salt extract turned out to be extremely unstable. It was rapidly lost upon attempts to fractionate it further. Moreover, the activity decreased upon storage in the cold, rapid freezing/thawing, or incubation at 37°C (Fig. 2, bar 9 vs. bar 11). Some preparations were more active than others (Fig. 2, bar 7 vs. bars 5 and 9), whereas some were completely inactive (Fig. 2, bar 3).

While testing whether small concentrations of the nonionic detergent Nikkol would be compatible with our assay system, we observed that the detergent increased the activity of crude

Table 1. Purification of the salt-extracted translocation activity

Fraction	Fig. 3, lane	ml	A_{280}/ml	A_{280}	Units/ml	Units	Units/A_{280}	Recovery, %	Enrichment
RM	a	20	50	1000	1000	20,000	20	100	1
Washed RM	b	20	31	620	1000	20,000	32	100	1.6
Salt extract	e	30	2.4	72	650	19,000	270	98	13.5
Salt extract (postribosomal)	f	24	0.95	22.8	450	10,800	474	54	23.7
ω-Aminopentyl-agarose eluate	i	2	0.21	0.42	5500	11,000	26,190	55	1310

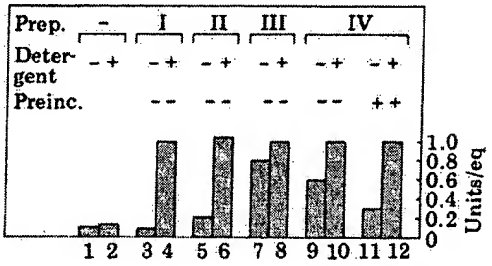

FIG. 2. Effect of low detergent concentrations on the activity of the salt extract. Translation (25 μl) was performed in the presence of 1 eq of salt-extracted washed RM. Crude salt extracts from four different preparations (I–IV) were added in increasing amounts. An aliquot of the salt extract (IV) was preincubated for 1 hr at 37°C in the absence or presence of 0.01% Nikkol. The dilution of the salt extract into the translation mix decreased the detergent concentration to 0.002%. Translations were carried out in the absence (−detergent) or presence (+detergent) of 0.002% Nikkol. The activity (units/eq) was computed from the percentage processing values.

salt extracts up to the theoretically expected level of 1 unit/eq (Fig. 2, bar 7 to bar 8 and bar 9 to bar 10). The detergent also stabilized the activity during an incubation for 1 hr at 37°C (Fig. 2, bar 10 to bar 12) and even reactivated preparations previously considered inactive (Fig. 2, bar 3 to bar 4 and bar 5 to bar 6). At the concentration employed (0.002%), the detergent by itself does not disrupt the lipid bilayer (Fig. 2, bar 2). Its concentration is one order of magnitude below the concentration needed to expose signal peptidase. PRL molecules translocated in the presence of 0.002% Nikkol are still completely protected from added proteases (data not shown).

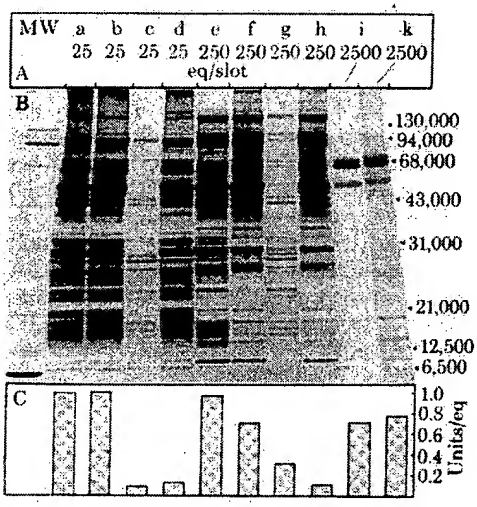

FIG. 3. Purification of the translocation activity monitored by polyacrylamide gel electrophoresis in $NaDodSO_4$. Fractions obtained during the purification of a typical preparation (lanes a–i) were Cl_3CCOOH-precipitated and their polypeptide composition visualized by Coomassie blue staining after electrophoresis in $NaDodSO_4$. (*A*) In lanes e–h 10 times more sample and in lanes i and k 100 times more sample (as defined in eq) is loaded relative to lanes a–d. (*B*) Lanes: MW, molecular weight standards; a, RM; b, washed RM; c, included fraction of the Sepharose C1-2B column; d, salt-extracted washed RM; e, salt extract; f, salt extract (ribosome-depleted); g, ribosomal pellet; h, fraction not bound to the ω-aminopentyl-agarose; i, eluate of the ω-aminopentyl-agarose; k, eluate of the ω-aminopentyl-agarose of another preparation of salt extract. (*C*) Activity of the fraction quantitated as described in Fig. 2. Membranous fractions (bars a, b, and d) were assayed directly, and soluble fractions (bars c and e–k) were assayed for their ability to reconstitute 1 eq of salt-extracted RM. Translations were carried out at a final concentration of 0.002% Nikkol.

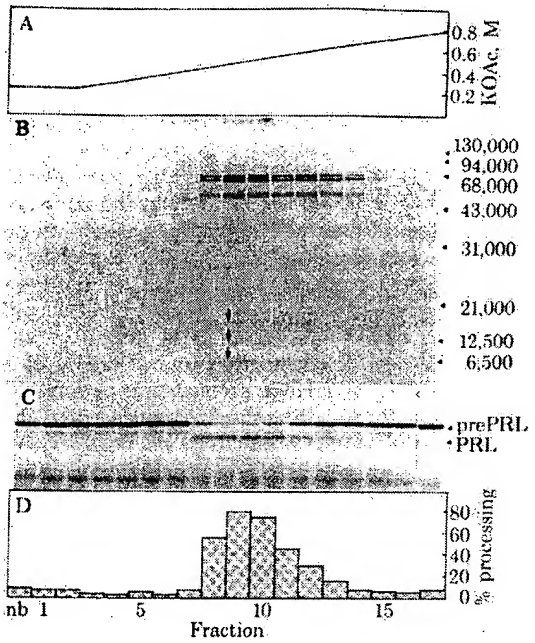

FIG. 4. DEAE-Sepharose ion-exchange chromatography of the ω-aminopentyl-agarose eluate. ω-Aminopentyl-agarose eluate (1.6 ml) was added to 4.8 ml of 50 mM $(EtOH)_3N$·HOAc/1 mM dithiothreitol. The sample was applied to a 1.5-ml column of DEAE-Sepharose C1-6B equilibrated in buffer D [50 mM $(EtOH)_3N$·HOAc/250 mM KOAc/2.5 mM $Mg(OAc)_2$/1 mM dithiothreitol/0.01% Nikkol] and a fraction of material not bound (nb) was collected. After loading, the column was washed with 4.5 ml of buffer D and eluted with 15 ml of a linear salt gradient of buffer D to buffer C (except that buffer C contained 0.01% Nikkol); 750-μl fractions were collected. (*A*) Addition of $[^3H]OAc^-$ to the gradient buffers allowed determination of the precise ion composition of each fraction. To 10 μl of each fraction, 5 μl of salt solution was added to yield a final ion concentration of 500 mM KOAc and 5 mM $Mg(OAc)_2$; 5 μl of these compensated fractions were assayed for activity (see Fig. 2). (*B*) Each fraction (300 μl) was Cl_3CCOOH-precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in $NaDodSO_4$. Arrows are used to indicate the position of the fainter bands of lower M_r. (*C*) Translation products. (*D*) Quantitative analysis of *C*.

Furthermore, the detergent does not interfere with the translocation activity of RM or washed RM preparations (data not shown).

These findings indicated that the rapid loss of activity was not an irreversible process but was merely caused by conversion of activity from a patent to a latent state. Moreover, the ability of nonionic detergent to restore activity to the theoretically expected level provided important clues concerning the properties of the active component and, thereby, opened the way to its complete and rapid purification.

To enrich the active component, the crude salt extract (without the addition of Nikkol) was centrifuged to remove ribosomes and ribosomal subunits. The activity present in both the supernatant and the ribosomal pellet was then assayed in the presence of Nikkol. From the total activity in the crude salt extract (Fig. 3*C*, bar e) and its quantitative partition (after centrifugation) into ribosome pellet (Fig. 3*C*, bar g) and into ribosome-depleted supernatant (Fig. 3*C*, bar f), it is apparent that about 30% of the activity sedimented into the ribosome pellet and that about 70% remained in the ribosome-depleted supernatant. Sedimentation of the activity with ribosomes is most likely because of its relatively high sedimentation coefficient (see below).

On the premise that the detergent stabilization of the active component might be caused by amphiphilic interactions, we

decided to use hydrophobic chromatography as a means of exploiting this peculiar property of the activity. We investigated the behavior of the activity on ω-aminoalkyl-agarose columns (12). The activity was not bound to ω-aminopropyl- and ω-aminobutyl-agarose but bound to ω-aminopentyl- and ω-aminohexyl-agarose in buffer B (data not shown). Elution of the activity by raising the salt concentration and including small concentrations of detergent in the buffer allowed 100% recovery from the ω-aminopentyl-agarose but only poor recovery from the ω-aminohexyl-agarose. Because most of the applied proteins were found not to interact with the ω-aminopentyl-agarose (Fig. 3*B*, lane h) a 55-fold purification was obtained in this step (see Table 1).

Three polypeptides of high M_r (72,000, 68,000, and 54,000) and three polypeptides of low M_r (19,000, 14,000, and 9000) were reproducibly obtained in the eluate (Fig. 3*B*, lane i). Some preparations contained contaminating bands (Fig. 3*B*, lane k) that did not appear consistently (Fig. 3*B*, lane i) and did not copurify with the activity upon subsequent sucrose gradient centrifugation, ion-exchange chromatography, or adsorption chromatography. The staining intensity with Coomassie blue of the three low M_r bands was slightly variable from gel to gel.

The substantially purified fraction obtained after hydrophobic chromatography was subjected to further fractionation using gel filtration on Sepharose Cl-6B (data not shown), ion-exchange chromatography on DEAE-Sepharose Cl-6B (Fig. 4), and adsorption chromatography on hydroxyapatite (Fig. 5). All six polypeptides consistently cochromatographed with

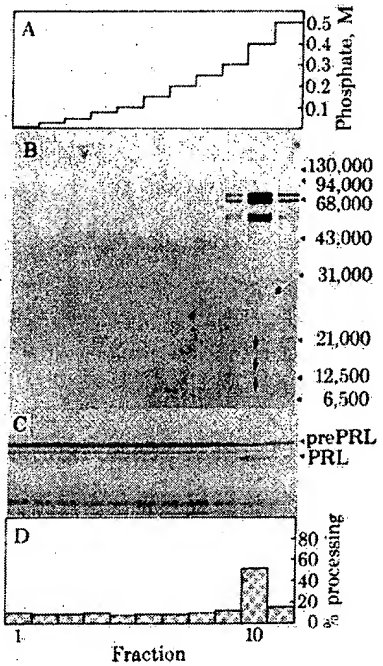

FIG. 5. Adsorption chromatography of the ω-aminopentyl-agarose eluate on hydroxyapatite. ω-Aminopentyl-agarose eluate (0.5 ml) was salt-exchanged on a 2.5-ml Sephadex G-25 column into a 10 mM sodium phosphate buffer (pH 6.5) containing 0.01% Nikkol and was applied to a 0.5-ml hydroxyapatite column. (*A*) The column was eluted with a step gradient of increasing phosphate concentration (1 ml per step). The phosphate buffer of each fraction collected was exchanged for buffer B (containing 0.01% Nikkol) on a 5-ml Sephadex G-25 column. (*B*) Each fraction (840 μl) was Cl_3CCOOH-precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in $NaDodSO_4$. (*C*) Each fraction (5 μl) was then assayed for translocation activity (see Fig. 2). (*D*) Quantitative analysis of the translation products in *C*.

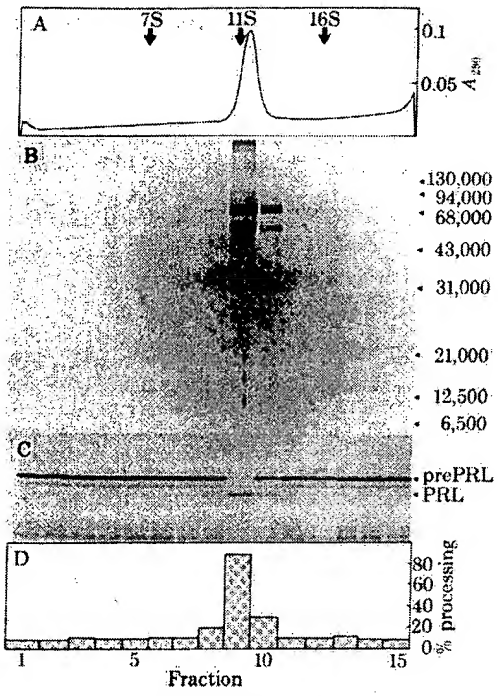

FIG. 6. Sucrose gradient centrifugation of the ω-aminopentyl-agarose eluate. ω-Aminopentyl-agarose eluate (250 μl) was diluted with an equal volume of water, was layered on top of a 5–20% sucrose gradient (13 ml) in buffer B containing 0.01% Nikkol, and was centrifuged for 20 hr at 4°C at 40,000 rpm in the Beckman SW 40 rotor. Fifteen fractions were collected using an ISCO gradient fractionator. (*A*) The A_{280} profile of the gradient with the positions of standards human IgG (7S), catalase (11S), and β-galactosidase (16S). (*B*) The remainder of each fraction was Cl_3CCOOH-precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in $NaDodSO_4$. (*C*) Each fraction (5 μl) was assayed for translocation activity (see Fig. 2). (*D*) Quantitative analysis of the translation products in *C*.

the activity, which strongly suggests that the activity consists of a complex containing six subunits.

When centrifuged on a sucrose gradient, the activity (containing all six bands) was found to sediment at about 11S (Fig. 6). This sedimentation rate indicates a molecular weight of about 250,000, in good agreement with the sum of the apparent M_r of the six subunits (236,000) as estimated by electrophoresis in $NaDodSO_4$/polyacrylamide gels.

The only biochemical characterization of the translocation activity that had previously been achieved is the observation that it can be inactivated by *N*-ethylmaleimide and, therefore, most likely requires a sulfhydryl group(s) for activity (8). We inactivated our purified preparation with *N*-[^{3}H]ethylmaleimide under conditions that had previously been shown (8) to inactivate the salt- or trypsin-extracted activity (Fig. 7, bar D vs. bar E). An autoradiograph of the inactivated preparation reveals that the M_r 68,000 and the 9000 bands were labeled intensely, the M_r 54,000 band was labeled less efficiently, and the M_r 19,000 band was labeled to trace amount. No label was detected in the M_r 72,000 and 14,000 bands.

DISCUSSION

We have described here the purification of a protein component involved in the cotranslational translocation of nascent presecretory proteins across the membrane of the ER. This component is associated with the ER membrane of dog pancreas and can be extracted from the membranes by high ionic strength buffers (7, 8), thereby largely depleting the vesicles of their

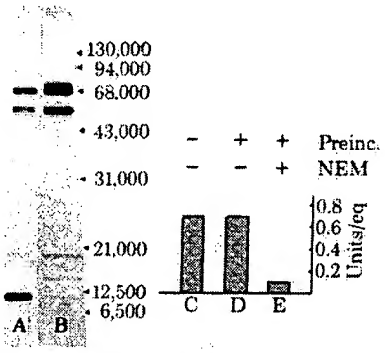

FIG. 7. Labeling of the ω-aminopentyl-agarose eluate with *N*-[^{3}H]ethylmaleimide. ω-Aminopentyl-agarose eluate (500 μl) was salt-exchanged on a 2.5-ml Sephadex G-25 column into 1 ml of 10 mM sodium phosphate buffer (pH 6.5) containing 0.01% Nikkol and 150 mM KOAc. Aliquots (475 μl) were then incubated in the absence (−) and in the presence (+) of 237.5 nmol (125 μCi; 1 Ci = 3.7×10^{10} becquerels) of *N*-[^{3}H]ethylmaleimide (NEM) for 30 min at 25°C (8). Dithiothreitol (9.5 μl of 0.5 M) was added to terminate the reaction. Aliquots (50 μl) were then salt-exchanged on 250-μl Sephadex G-25 superfine columns into buffer B (containing 0.01% Nikkol) and were assayed for translocation activity (see Fig. 2) (bars C, D, and E). Aliquots (425 μl) of the incubated samples were Cl_3CCOOH-precipitated and subjected to electrophoresis in $NaDodSO_4$. The gel lane containing the *N*-[^{3}H]ethylmaleimide-treated aliquot was fluorographed (lane A), whereas the gel lane containing the nontreated aliquot was stained with Coomassie blue (lane B).

translocation activity. Reconstitution of the translocation activity by adding salt extract back to the extracted vesicles (7, 8) provided a convenient assay to monitor the purification of the active component present in the extract. We found that hydrophobic chromatography of the ribosome-depleted salt extract allowed essentially a one-step purification of the active protein component.

When analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$, the purified protein was composed of six polypeptides. Because these six polypeptides were not separated from each other by several nondenaturing chromatographic procedures, they most probably form a complex with a molecular weight of about 250,000, as judged by sedimentation analysis and summation of the estimated size of the individual subunits. However, we cannot rule out the possibility that one or more of the six polypeptides become associated with the activity during isolation.

The mode of interaction of the complex with the membrane and its relationship to a functionally equivalent activity that can be extracted (even at low salt concentration) after limited proteolysis of the membranes (6, 8) remain to be established. However, a few conjectures on its possible mode of action can be made, based on its quantitatively estimated relationship to the number of translocated chains, its labeling behavior with its inhibitor *N*-ethylmaleimide, and its observed interactions with hydrophobic moieties.

We have estimated (data not shown) that 3 to 10 fmol of PRL are translocated per round of translation in 25 μl of our *in vitro* assay containing 1 eq of RM. We furthermore estimated (from the Coomassie blue staining intensity) that 1 eq of purified complex consists of about 20 fmol. Therefore, these estimates suggest a stoichiometric relationship within one order of magnitude between the number of complexes and the number of translocated chains. Consequently, the complex is likely to be required stoichiometrically rather than catalytically for each chain translocation event.

Incubation with *N*-[^{3}H]ethylmaleimide labeled two of the six subunits intensely under conditions that abolish its activity. Because a sulfhydryl group requirement for the activity of the unfractionated salt extract has been demonstrated (8), these results suggest that the sulfhydryl group(s) in question locate to either one or two subunits of the complex.

The purification was facilitated by the observation that the activity can be stabilized and, in fact, can be reactivated by low concentrations of nonionic detergent. This permitted quantitation of the activity at each step of the purification. If one takes into consideration that the complex is essentially the only component of the salt extract that interacted with the hydrophobic matrix of the ω-aminopentyl-agarose, it is conceivable that the exposed hydrophobic region of the complex is important in the recognition of the hydrophobic portion of the signal sequences or, alternatively, in anchoring of the complex to the hydrophobic core of the lipid bilayer.

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1. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* 67, 835–851.
2. Blobel, G., Walter, P., Chang, C. N., Goldman, B. M., Erickson, A. H. & Lingappa, V. R. (1979) *Society for Experimental Biology Symposium*, eds. Hopkins, C. R. & Duncan, C. J. (Cambridge Univ. Press, Great Britain), Vol. 33, pp. 9–36.
3. Blobel G. & Dobberstein, B. (1975) *J. Cell Biol.* 67, 852–862.
4. Szczesna, E. & Boime, I. (1976) *Proc. Natl. Acad. Sci. USA* 73, 1179–1183.
5. Jackson, R. C. & Blobel, G. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5598–5603.
6. Walter, P., Jackson, R. C., Marcus, M. M., Lingappa, V. R. & Blobel, G. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1795–1799.
7. Warren, G. & Dobberstein, B. (1978) *Nature (London)* 273, 569–571.
8. Jackson, R. C., Walter, P. & Blobel, G. (1980) *Nature (London)* 284, 174–176.
9. Scheele, G., Dobberstein, B. & Blobel, G. (1978) *Eur. J. Biochem.* 82, 593–599.
10. Shields, D. & Blobel, G. (1978) *J. Biol. Chem.* 253, 3753–3756.
11. Hawkins, H. C. & Freedman, R. B. (1979) *Biochem. Biophys. Acta* 558, 85–98.
12. Shaltiel, S. (1974) *Methods Enzymol.* 34, 126–140.
13. Lingappa, V. R., Devillers-Thiery, A. & Blobel, G. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2432–2436.
14. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* 70, 2330–2334.
15. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* 67, 247–256.